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ISOLATION OF THE ADP, ATP CARRIER AS THE CARBOXYATRACTYLATE · PROTEIN COMPLEX FROM MITOCHONDRIA

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Summary

The procedure for the isolation from mitochondria of the undenatured ADP, ATP carrier is described. The condition of retaining the nativity are elaborated.

1. As indicator for the ADP, ATP carrier [^{35}S]- or [^{3}H]carboxyatractylate were used. By preloading the mitochondria with carboxyatractylate, a stable carboxyatractylate · protein complex could be retained after solubilization with Triton X-100. Among the polyoxyethylene detergents emulphogen is also solubilizing, whereas Brij and Lubrol fail to solubilize.

2. When unloaded mitochondria are solubilized the capacity for binding carboxyatractylate disappears rapidly, particularly at 20° C.

3. When mitochondria are preloaded with atractylate, the binding after solubilization with Triton X-100 is considerably lower than with carboxyatractylate, indicating that the high affinity of carboxyatractylate is required for effectively protecting the protein.

4. For purification hydroxyapatite is most effective. The carboxyatractylate-protein complex appears in the pass-through whereas the bulk of other mitochondrial proteins are retained such that a 7-fold purification is obtained. The nonadsorptivity to hydroxyapatite is dependent on the undenatured state maintained in the carboxyatractylate · protein complex.

5. Subsequent gel filtration on Sepharose results in a 1.5-fold further enrichment of specific carboxyatractylate binding up to 17 μmol/g protein, corresponding to a 10-fold purification from mitochondria. This value cannot be increased with further measures.

6. At the last purification step, in sodium dodecyl sulfate polyacrylamide gel electrophoresis virtually a single band of 30 000 molecular weight is found,

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Abbreviation: SDS, sodium dodecylsulfate

confirming the purity at this stage. A molecular weight of 60 000 is calculated from the carboxyatractylate binding, indicating that the carboxyatractylate protein complex consists of two 30 000 subunits. From this the protein share of the ADP, ATP carrier in beef heart mitochondria can be calculated to amount to 9.5%.

7. The intact carboxyatractylate · protein complex is protected against proteolytic degradation. The release of carboxyatractylate ensues a conformational change of protein as assayed by conformation specific antibodies, concomitant with unmasking of proteolytic site as assayed by tryptic digestion.

8. The amino acid composition indicates hydrophobicity (39% polarity) and a high content of basic amino acid such as lysine and arginine. There is 1.5 mol percent cysteine and a blocked N-terminal.

9. From the solubilized complex [^{35}S]carboxyatractylate can be removed by carboxyatractylate, ADP and ATP but not by ITP, etc., indicating the presence of recognizing sites specific for ADP, ATP and therefore, identity with the ADP, ATP carrier.

10. Other reported procedures for isolating the ADP, ATP carrier are shown to either fail or have lower yield than the present, original procedure.

Introduction

The inner mitochondrial membrane contains a most active and highly specific system for facilitating the exchange between intra- and extramitochondrial ADP and ATP (for review see ref. 1). This transport could be expected to be catalyzed by a specific membrane protein, the characterization of which was an aim of our research since the discovery of this transport system in 1964 [2]. A first step towards this goal was the detection of the carrier sites at the membrane by specific binding with ADP, ATP [3] and with the inhibitors atractylate [4,5], carboxyatractylate and bongkrekate [7,8]. Among these, carboxyatractylate appeared to be the best candidate for labelling the carrier protein during solubilization from the membrane because of its high affinity. The highly specific, noncovalent binding of carboxyatractylate signifies that after isolation the protein is not denatured, which was an important postulate from the onset of our isolation work. ADP and ATP as markers should be less suitable because of their lower affinity and the high sensitivity to perturbations of the membrane [3].

The usual procedure of applying this ligand for identifying the carrier protein after solubilization would be to measure the binding of isotope labelled carboxyatractylate on addition to the solubilized fractions. This procedure has been studied by us in detail for solubilization of the membrane protein with a number of ionic and nonionic detergents [9—11]. However, in this manner the carrier protein could not be identified since such preparations expose a large number of unspecific binding sites for carboxyatractylate obviously as a result of partial denaturation [9]. It would also be difficult to detect thus the less numerous specific binding sites for carboxyatractylate.

As a results we changed the procedure in order to avoid the interference of unspecific binding such that carboxyatractylate was given first to the intact

membrane where it was known to bind only to specific carrier sites [9.10]. As an additional most important benefit, prior binding of carboxyatractylate to the protein turned out to protect the carrier against inactivation during the isolation procedure.

This isolation method was first reported in 1974 [9] and then described in a brief communication [10,11]. Subsequent to our first report, publications on the isolation of the ADP, ATP carrier from other laboratories using largely different procedures appeared [12—14]. It will be shown in this paper that these methods either fail or they produce the carrier with only low yield.

In view of the great importance of this protein there is an urgent need to clarify the problem of isolation and give a full account of the procedures, thus making available the highly efficient and simple process of preparing this most abundant membrane protein.

Materials and Methods

Triton X-100 was obtained from Sigma Chemical Corp., the Brij series from Atlas Corp., Emulphogen BC 720 from GAF Corp., New York, carboxyatractylate from Boehringer, Mannheim, Sepharose 4B and 6B from Pharmacia, hydroxylapatite ("HTP") from Bio-Rad Laboratories or prepared as described elsewhere [23].

[^{35}S]Carboxyatractylate was isolated from roots of atractylis gummifera as described earlier [15]. [^{3}H]Carboxyatractylate was synthesized from commercially available carboxyatractylate (Babel, W., unpublished). Beef heart mitochondria were isolated as described by Smith [16]. Protein content was determined by a modified biuret [17] or Lowry method in the presence of 0.5% SDS [18]. Bovine serum albumin was used as standard. [^{35}S]Carboxyatractylate binding to the solubilized protein was determined by equilibrium dialysis using the 200 μl cells of the "Dianorm" apparatus, manufactured by Dr. Weder (ETH, Zürich). Dialysis was run at room temperature for 3 to 6 h.

Polyacrylamide gel electophoresis in SDS was performed essentially with gels of 8% or 10% acrylamide monomer and 0.35% *N,N*-methylenbisacrylamide in the presence of 0.1% SDS 5 × 60, ×150 or ×240 nm gels were used. Usually 1 to 1.5 μg protein per band was dissolved in 2 or 4% SDS, 10 mM phosphate, 5% sucrose, 2 or 4 mM dithioerythrol at pH 7.2 and heated to 100°C for about 2 min before applied to the gels. Staining was carried out by Coomassie brilliant blue G-250.

For electrophoresis and amino acid analysis Triton X-100 was partially extracted from the protein solution with diethylether or acetone.

Results

Solubilization

Beef heart mitochondria first loaded with [^{35}S]carboxyatractylate up to saturation of all binding sites were treated with a number of different detergents or solvents for solubilizing the [^{35}S]carboxyatractylate binding protein. In extracts using ionic detergents such as SDS, deoxycholate and cholate, the released carboxyatractylate was found to be free, using the dialysis assay.

Obviously the protein becomes denatured with these detergents to such an extent that it loosens the bound carboxyatractylate. Also chaotropic and H-bond splitting reagents such as guanidine/HSCN and urea, and membrane solvents such as chloroethanol released free carboxyatractylate from the membrane.

In contrast, some nonionic detergents were effective solubilizers of the intact carboxyatractylate · protein complex. The solubilization by some polyglycol detergent homologues is compared in Fig. 1. Most effective is Triton X-100 containing octylphenyl as a lipophilic group. Also Emulphogen BC 720, with an isotridecyl moiety, solubilizes the carboxyatractylate protein although being considerably less effective. Brij 58 and Lubrol WX with unbranched alkyl chains are unable to solubilize the carboxyatractylate · protein complex.

The influence of salt on the solubilization is shown in Fig. 2, as a function of increasing NaCl concentration at three different Triton X-100 : protein ratios. A remarkably synergistic effect of detergent and salt is observed such that less Triton is required with higher salt concentration. It is not quite clear at present whether the salt augments the solubilizing power of the detergent or the membrane structure becomes more amenable to detergents, for example by loosening ionic bonds of the phospholipid headgroups, in particular those of cardiolipin. Addition of salts (KCl, $(NH_4)_2SO_4$) have been used frequently by other authors for solubilizing mitochondria with cholate.

The efficiency of using carboxyatractylate instead of atractylate as labels

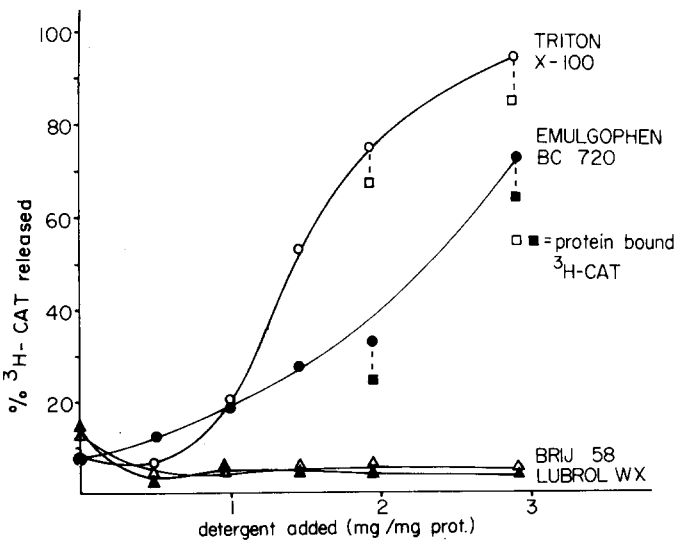

Fig. 1. The effect of various polyoglycol detergents on the solubilization of the carboxyatractylate · protein complex. Mitochondria preloaded with [^{3}H]carboxyatractylate (CAT) were incubated at 5.1 mg protein/ml, increasing concentrations of detergent, reaching 6% at maximum. The apparent compositions of the detergents are: (R-(EO)$_n$, EO = ethylenoxide group), Triton X-100 = pt-octylphenol-(EO)$_{9-10}$, Emulphogen BC = isotridecyl-(EO)$_{12}$, Brij 58 = cetyl-(EO)$_{20}$, Lubrol WX = cetyl-, stearyl-(EO)$_{17}$. The medium contained in addition 0.4 M NaCl, 20 M Tris, pH 7.4, 0°C. Supernatants of 40 000 rev./min centrifugation were measured for [^{3}H]carboxyatractylate content. Binding of [^{3}H]carboxyatractylate protein was measured by equilibrium dialysis (see Methods).

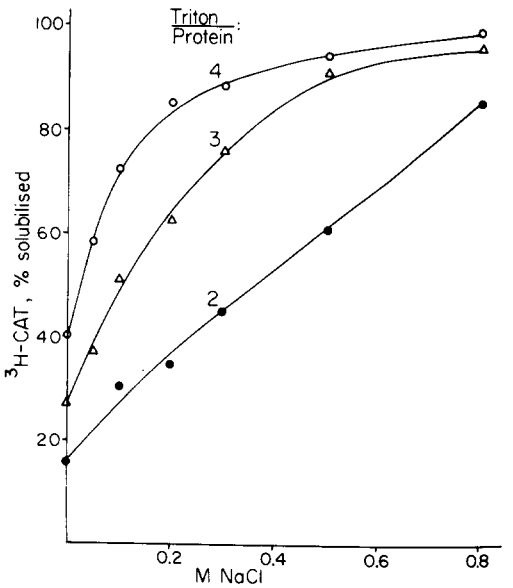

Fig. 2. Dependence of solubilization of [^{3}H]carboxyatractylate · protein complex on salt concentration. [^{3}H]Carboxyatractylate (CAT) loaded beef heart mitochondria were incubated with Triton/protein weight ratios = 2, 3 and 4 as indicated, and increasing concentration of NaCl, as shown by the abscissa. Incubation at pH 7.2 at 0°C in 10 mM morpholinopropane sulfonic acid. Supernatants of 40 000 rev./min were measured.

for the solubilized protein was also investigated. Atractylate is retained after solubilization only between 30 and 60% whereas carboxyatractylate binds to 90—100%. This indicates that the lower binding affinity to atractylate known from the intact membrane is too weak to survive fully the solubilization by Triton X-100. Therefore, by using [^{35}S]atractylate one cannot quantitatively identify the isolated protein. This also explains why the use of [^{3}H]-atractylate by Brandolin et al. [12] has not given satisfactory results.

The binding capacity for carboxyatractylate was found to be decreased and completely destroyed after solubilization with Triton if it is not added prior to the detergent. The experiment in Fig. 3 shows the disappearance of the binding capacity for carboxyatractylate after addition of Triton to the mitochondria. The inactivation is strongly accelerated at 20°C as compared to 0°C. At 0°C there is first a rapid inactivation up to 2 min, followed by a relatively slow phase. The relatively stable level of binding capacity up to 100 min at 0°C corresponds to about half of the original capacity. It can be assumed that at least at this stage the carrier protein is fully solubilized whereas the initial rapid inactivation may coincide with the solubilization process. The two phase kinetics raise a number of questions on the nature of the inactivation process of the unloaded carrier protein which go beyond the scope of the present paper and will be dealt with in later reports.

As reported elsewhere [19] unloaded carrier protein was isolated with extended stability using an aminoxide type detergent. This protein preparation has been used in reconstitution studies, for incorporating the carrier protein into phospholipid vesicles.

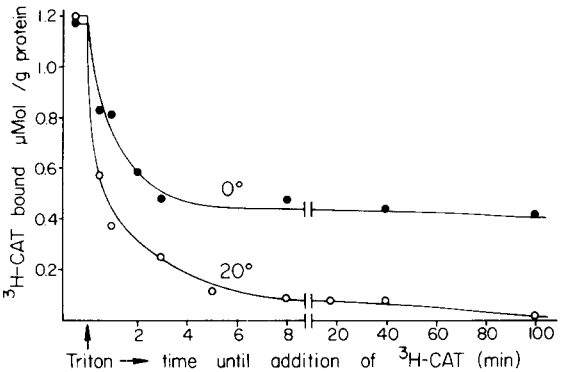

Fig. 3. The loss of carboxyatractylate (CAT) binding capability in Triton extracts from beef heart mitochondria. 5 mg protein/ml mitochondria incubated at pH 7.2, 0°C, in 0.6 M NaCl, 50 M morpholinopropane sulfonic acid. At time zero, Triton is added and samples are withdrawn and rapidly mixed with 5 µM [^{3}H]carboxyatractylate. After 50 min the extracts were centrifuged at 40 00 rev./min for 60 min and then given to Sephadex G-75 columns for measuring the amount of [^{3}H]carboxyatractylate bound. For measuring the binding without Triton, one sample was withdrawn before addition of Triton, mixed with carboxyatractylate and then extracted with Triton X-100. Each series was run at 0°C and 20°C.

Purification

With the successful solubilization of the carboxyatractylate · protein complex, a starting point for the purification was found. Among several purification methods tested, such as chromatography on various media, electrofocussing, etc., essentially two procedures turned out to be most efficient: gel chromatography on Sepharose 6B and adsorption chromatography on hydroxyapatite.

The gel chromatogram of one example of solubilized extract is shown in Fig. 4. Here mitochondria are first extracted with cholate in order to remove other protein, under conditions which do not solubilize the carboxyatractylate protein. The residue is solubilized with Triton X-100 and applied to Sepharose 6B column. Nearly all the [^{35}S]carboxyatractylate is collected in the medium molecular weight range, widely separated from the region where free [^{35}S]-carboxyatractylate would appear. At the peak fraction, 8 µmol carboxyatractylate/g protein are found corresponding to an about 5-fold purification from enriched membranes. With these fractions a further purification can be obtained by subsequent hydroxyapatite chromatography.

The adsorption chromatography on hydroxyapatite or calcium phosphate gel for the fractionation of the detergent solubilized extracts proved to be of great advantage. In particular, if the adsorption chromatography on hydroxyapatite is applied first to the crude extract, a rapid and high degree of purification is obtained in one step (Fig. 5). The carboxyatractylate · protein complex is confined to the pass-through whereas most of the other solubilized mitochondrial proteins are adsorbed and thus eluted by a phosphate gradient. No cytochochromes are found by difference spectrophotometry in the pass-through. Cytochrome *b* and c_1 have been found in later work to be eluted as a cytochrome bc_1 complex by phosphate in well defined fractions [20,21].

The amount of protein which appears in the pass-through of the hydroxyapatite column from crude extracts of Triton solubilized mitochondria is

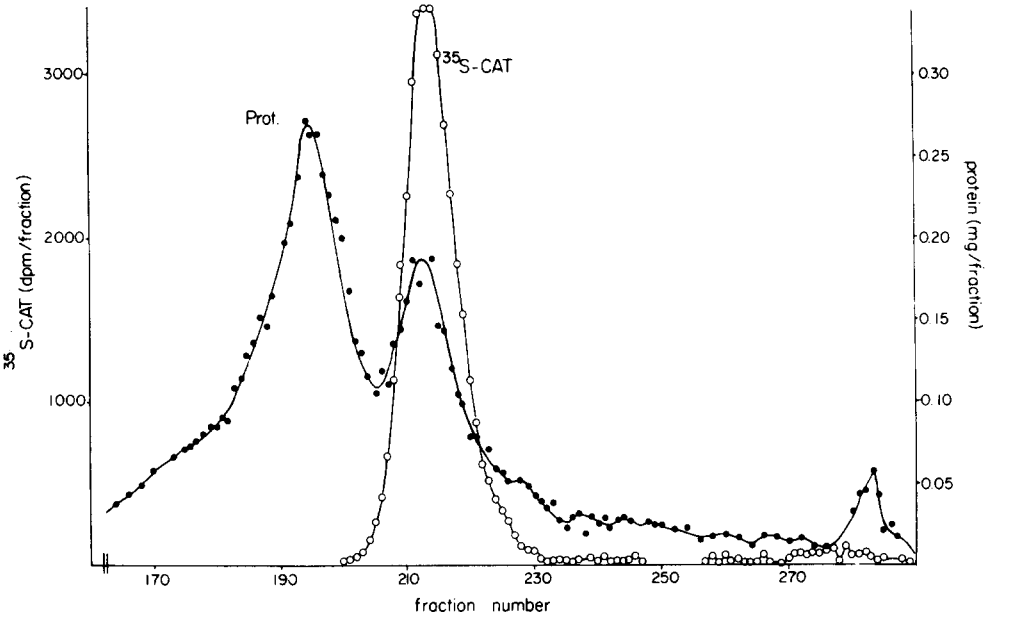

Fig. 4. Gel filtration on "Sepharose 6B" column of a crude Triton X-100 extract from beef heart mitochondria loaded with [^{35}S]carboxyatractylate. Beef heart mitochondria are incubated with 1.86 μmol [^{35}S]carboxyatractylate/g protein and washed by centrifugation. The residue was solubilized with Triton X-100 and about 8.2 mg protein applied to the column. Elution with 1% Triton X-100, 100 mM NaCl, 0.25 mM EDTA, pH 7.2.

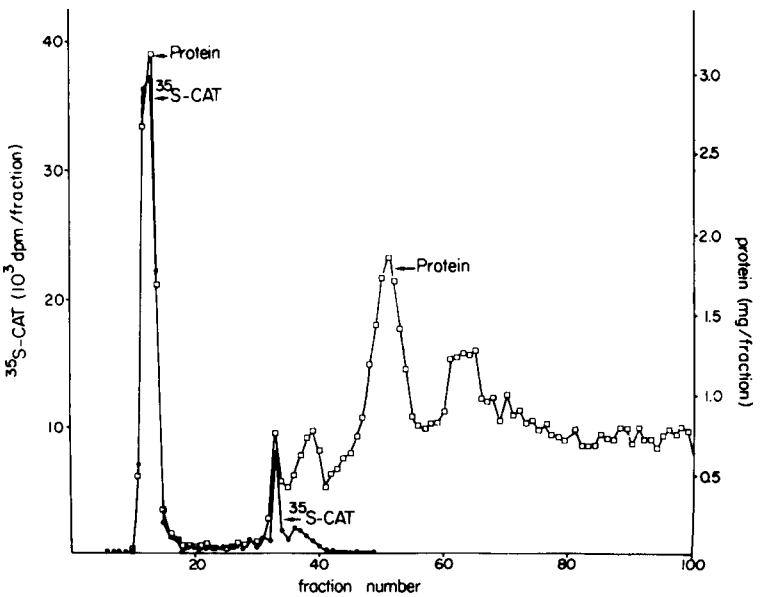

Fig. 5. Adsorption chromatography on a hydroxyapatite column of a Triton X-100 extract of mitochondria saturated with [^{35}S]carboxyatractylate. 1 mg protein was applied in 60 μl extract volume per ml of packed hydroxyapatite. Proteins were eluted first with 0.5% Triton X-100, 10 mM morpholinopropane sulfonic acid, 100 mM NaCl, pH 7.2 (Eluent I). After fraction 13, a linear gradient (14 ml/ml hydroxyapatite) up to 0.2 M phosphate was applied in order to elute other proteins. A subsequent elution increasing up to 0.4 M phosphate is not shown. Flow rate: 10.4 ml/h, 5.1-ml fractions.

dependent on loading with carboxyatractylate [22]. A relatively smaller amount of protein passes through the column in extracts from unloaded mitochondria. With ADP the increase is only minor. Also by loading with bongkrekate the elution of the protein is promoted [23]. When extracted from unloaded mitochondria, this protein becomes largely adsorbent to hydroxyapatite concomitant with the loss of binding for carboxyatractylate. It may be concluded that the undenatured form as retained in the carboxyatractylate · protein complex is nonadsorbent to hydroxyapatite.

For further purification the pass-through from the hydroxyapatite column can be applied to gel-chromatography on agarose colums (Sepharose 6B or Ultrogel AcA 34) (Fig. 6). The carrier is distinctly enriched in the middle of a peak as shown by the carboxyatractylate : protein ratio. Occasionally this peak is trailed by a pronounced shoulder of carboxyatractylate · protein and the major amount of Triton appears after the protein peak. In other experiments (not shown) it was observed that also the phospholipid is eluted at a maximum coincident with Triton. The experience shows that with extracts containing higher amounts of Triton, the filtration process can become congested by Triton micelles and retard protein elution [24]. This may explain the shoulder formation of the carboxyatractylate protein wich consists of the same polypeptide as the main peak (see Fig. 3) [15]. In fact, the carboxyatractylate/protein ratio in this shoulder is the same and indicates homogeneity with the main peak.

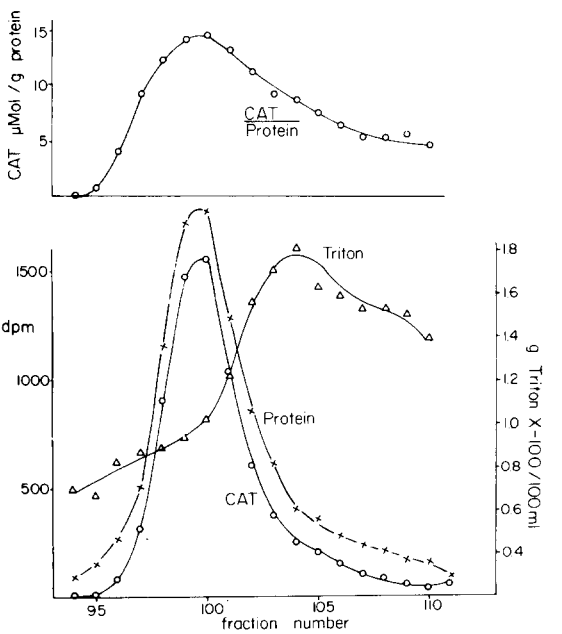

Fig. 6. Gel filtration on agarose/acrylamide gel (type AcA 34) of the pass-through from a hydroxyapatite column. The chromatogram is derived from a column for large scale preparation containing 2 liters of AcA 34. The Triton X-100 extract of 2 g mitochondrial protein (see Results) are added to the column and eluted with a medium containing 0.1 M Na_2SO_4, 0.5% Triton X-100, pH 7.2. Each fraction contained 21 ml.

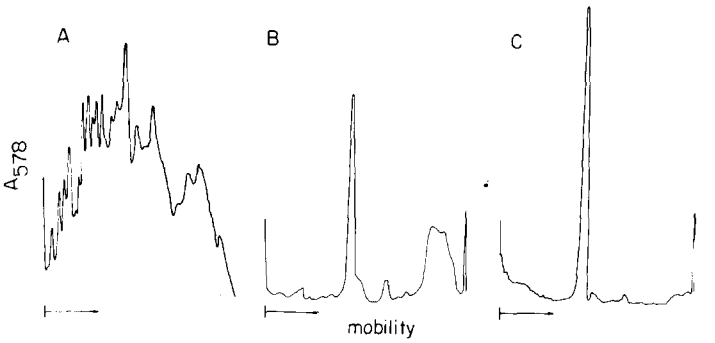

Fig. 7. SDS-polyacrylamide gel electrophoresis of the purification stages. (a) Triton X-100 extract of mitochondria preloaded with carboxyatractylate (CAT). (b) Pass-through of the hydroxyapatite columns (Fig. 5). (c) [^{35}S]carboxyatractylate binding protein after gel filtration on Sepharose 6B.

The various purification steps were assayed by polyacrylamide gel-electrophoresis in SDS (Fig. 7). Already in the pherogram of the crude extract from carboxyatractylate loaded beef heart mitochondria an important band with a molecular weight of 30 000 stands out. After passing through the hydroxyapatite column this band is drastically enriched. An approximate purity of about 60% can be estimated from the gels. The peak fraction from gel filtration finally gives a single protein band in SDS gel electrophoresis without an apparent contamination. By calibration with other proteins a molecular weight of about 30 000 was determined.

The purity of the carboxyatractylate protein is assayed in terms of the relative amount of [^{35}S]carboxyatractylate bound. A maximum of 18 to 19 μmol carboxyatractylate/g protein can finally be obtained. The maximum enrichment is about 10-fold. All further attempts to purify this protein by using other chromatographic material such as DEAE-cellulose did not further increase the carboxyatractylate content.

The pellet enriched with the [^{35}S]carboxyatractylate binding protein was dissolved in a medium containing 4 to 5% Triton, 0.5 M NaCl at pH 7.2 and after 30 min the suspension was centrifuged for 60 min at 100 000 $\times g$.

The supernatant was then directly applied to hydroxyapatite column at no more than 1.5 mg protein/ml gel. The column was equilibrated with a solution containing 0.5% Triton, 100 mM NaCl at pH 7.2. The protein was eluted with the same medium.

The pass-through fractions were pooled, concentrated 2 to 3 times and applied to a Sepharose 6B column. The protein fractions showing the same number of binding sites for the bound [^{35}S]carboxyatractylate were finally pooled and checked for purity by electrophoresis (Fig. 7C).

Amino acid composition

The amino acid composition of the purified carboxyatractylate-binding protein is given in Table I. The high content of lysine and arginine is in agreement with the high isoelectric point (10.3) determined by isoelectric focussing. The polarity of 40% as calculated according to [25], corresponds to an only mildly hydrophobic character of the protein. In contrast to our previous

TABLE I

AMINO ACID COMPOSITION OF THE CARBOXYATRACTYLATE BINDING PROTEIN FROM BEEF HEART MITOCHONDRIA

Asx	7.4	Cys	1.69	Tyr	4.9
Thr	4.5	Met	2.6	Phe	6.7
Ser	5.0	Ile	5.6	His	0.8
Glx	7.1	Leu	8.1	Lys	7.5
Pro	3.0			Arg	5.3
Gly	10.9				
Ala	11.4				
Val	7.6				

report, now 1.2 to 1.7% cysteine is found by the performic acid oxidation procedure. In new measurements no free N-terminal is found, in correction to our previous report [11].

Proteolytic breakdown and denaturation of the carboxyatractylate-binding protein

Throughout the purification procedure the protein was maintained in an undenatured state by binding carboxyatractylate. Experiments show that the unprotected protein does not only lose its binding capability but also undergoes a proteolytic breakdown [23]. This degradation interferes with the isolation of the carrier protein if the carrier is unprotected. The proteolytic breakdown was investigated in more detail starting from the isolated carboxyatractylate · protein. For this purpose carboxyatractylate was removed from the purified protein by addition of ATP or ADP.

The release of [^{35}S]carboxyatractylate in dependence on the concentration of ATP and salt is shown in Fig. 8. The specificity of this effect is demonstrated by the fact that ITP is unable to remove carboxyatractylate. This

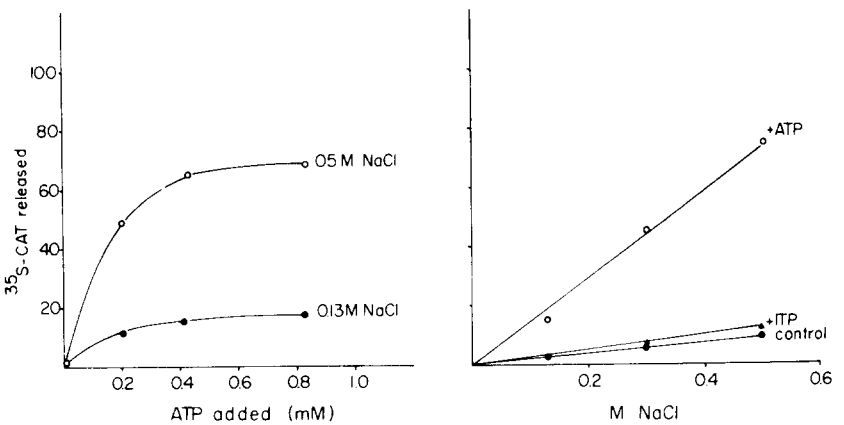

Fig. 8. [^{35}S]Carboxyatractylate (CAT) displacement in Triton X-100 extracts of [^{35}S]carboxyatractylate preloaded mitochondria by ATP. Dependence on concentration of ATP (A) and of NaCl (B). Equilibrium dialysis in 6% Triton X-100, 10 mM morpholinopropane sulfonic acid, NaCl as indicated, pH 7.2 at room temperature for 6 h. Addition of 0.83 mM ATP or 0.83 mM ITP.

result has been interpreted to show that the isolated carboxyatractylate · protein still has specific recognition site for ATP similar to the ATP, ADP carrier. It is therefore probable that the isolated [^{35}S]carboxyatractylate · protein comprises the ADP, ATP carrier, in which the affinity for ADP is strongly decreased such that binding of [^{3}H]ATP to the protein after releasing [^{35}S]carboxyatractylate cannot be measured (unpublished results).

The degradation after removal of [^{35}S]carboxyatractylate is shown on gel-chromatography (Fig. 9). A new protein peak with lower molecular weight appears which does not contain [^{35}S]carboxyatractylate. The amount of this protein corresponds to the release of about 50% of the [^{35}S]carboxyatractylate. The electrophoresis shows that the second peak consists of two polypeptide fractions of 25 000 and 20 000 mol. wt. These can only originate from the 30 000 protein by proteolytic breakdown.

It was then asked whether the ADP induced changes are caused first by a loss of the native conformation which ensues proteolytic degradation. In order to follow the unmasking of the proteolytic sites the protein is incubated in the presence of trypsin. As a specific assay for the original conformation, the antigenic reactivity with the conformation specific carboxyatractylate · protein · antibody was followed [26]. At increasing time after ADP addition, samples were withdrawn and analyzed for carboxyatractylate binding

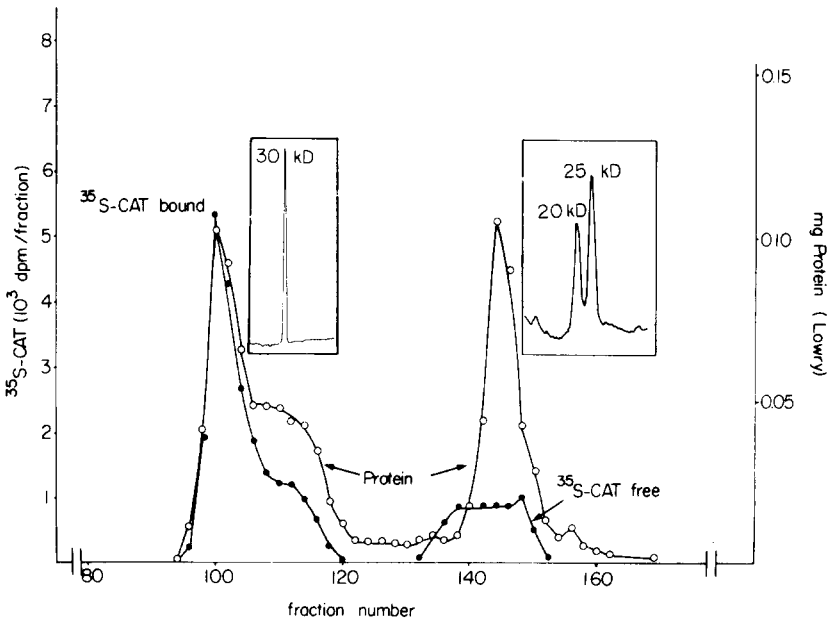

Fig. 9. Partial degradation of carboxyatractylate (CAT) binding protein by exposure to ADP. Purified carboxyatractylate-binding protein was labelled by exchange with added [^{35}S]carboxyactractylate to a final activity of $1.8 \cdot 10^5$ cpm [^{35}S]carboxyatractylate bound/mg protein. The protein solution was then dialyzed versus 25 volumes of a buffer containing 0.5 mM ADP, 0.018 mM diisopropylfluorophosphate, 500 mM NaCl, 2.9% Triton X-100, 5 mM morpholinopropane sulfonic acid at pH 7.2 for 30 h at 4°C. After this time 40% of bound carboxyatractylate was removed. The solution containing 2 mg carboxyatractylate-binding protein was applied to a 1 × 10 cm Sepharose 6B column and eluted with 0.5% Triton X-100, 50 mM NaCl, pH 7.2. The second peak accounts to about 36% of the protein. Inserts: densitometric traces of SDS gel electrophoresis: (A) fraction 100, (B) fraction 144.

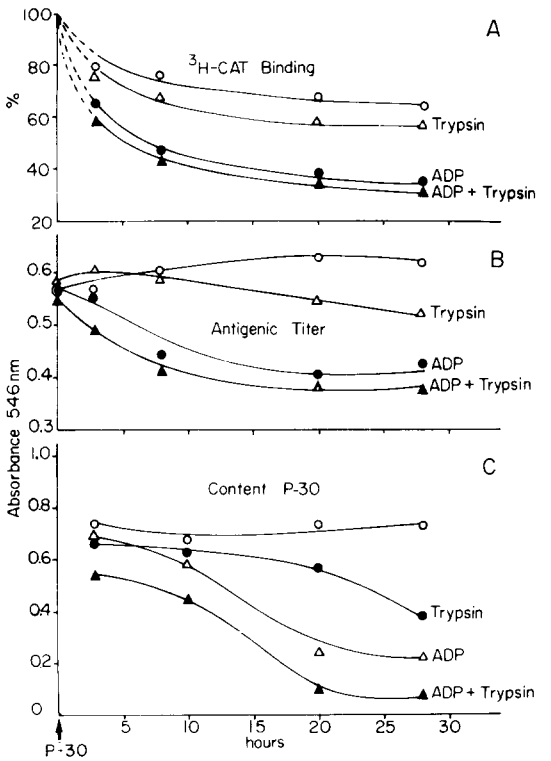

Fig. 10. Loss of conformational stability and exposure of proteolytic sites of removal of [^{3}H]carboxyatractylate (CAT) by addition of ADP. Purified [^{3}H]carboxyatractylate-loaded protein (400 μg protein) are incubated in 1 ml, containing 0.15 M Na_2SO_4, pH 7.0, at 24°C. If required, 8 μg trypsin are added. At time zero, 400 μM ADP are added, and at the time indicated in the abscissa sample (200 μl) are withdrawn and mixed with 0.8 μg trypsin inhibitor from soybeans. In aliquots of these samples (A) [^{3}H]-carboxyatractylate binding is determined by equilibrium dialysis. (B) The antigenic titer is determined by mixing equivalent of 16 μg protein with 0.13 mg antibody preparation (γ-globulin fraction). For this purpose both solutions are mixed in a cuvette in a photometer and turbidity increase is recorded at 546 nm. (C) The residual content of the 30 000 mol. wt. peptide (P-30) is determined by SDS gel electrophoresis.

(Fig. 10A), for the amount of 30 000 mol. wt. protein (Fig. 10C) and for the relative antigenic titer (Fig. 10B). After 20 h the [^{3}H]carboxyatractylate binding is decreased to 30% under the influence of ADP. Trypsin has only a small influence on the release of the [^{3}H]carboxyatractylate both alone or together with ADP. The antigenic titer follows quite closely the loss of [^{3}H]carboxyatractylate binding, indicating that the original "c"-conformation of the carrier has disappeared following the release of carboxyatractylate. It should be remembered that the loss of carboxyatractylate cannot be the cause for the disappearance of the antigenicity since it has been ruled out as a haptene [26,27] to the antibody. The disappearance of 30 000 mol. wt. protein (Fig. 10C) is strongly promoted by ADP. The proteolytic degradation is enhanced by trypsin, both in the presence and absence of ADP. The degradation is clearly delayed as compared to the loss of the antigenicity, indicating that the unfolding must precede the proteolytic digestion.

TABLE II

OTHER REPORTS FOR SOLUBILIZATION AND ISOLATION OF PROTEINS LINKED TO ADP, ATP CARRIER

Abbreviations: RLM, rat liver mitochondria; BHM, beef heart mitochondria; CAT, carboxyatractylate; ATR, atractylate.

Reference		Detergent	Reported results	Comments	Our results
Egan et al. [48]	RLM	Brij 58	Solubilization of ADP binding protein	No purification attempted	In these extracts no binding of ADP and [^{3}H]CAT can be found
Brandolin et al. [12]	RLM	Emulphogen BC 720	Solubilization and enrichment of ATR binding protein by affinity chromatography	Procedure used the protection method reported by us (8a). No identification of Mol. Weight of polypeptide in polyacrylamide gel electrophoresis. From gel chromatography 15 000 and 60 000 mol. wts. were estimated	Solubilization of 30% [^{3}H]CAT protein at condition
	BHM				Solubilization of only 10% protein (see Fig. 1). At higher concentration of Emulphogen solubilization of up to 80%
Bojanowsky et al. [13]	RLM	SDS	Solubilization and purification of CAT and ADP binding protein	Isolated fraction binds CAT and ADP, polypeptide mol. wt. 26 000, only diffuse band in polyacrylamide gel electrophoresis	No binding of CAT and ADP in SDS extract possible. Protein denatured
Shertzer et al. [14]	BHM	Cholate	Solubilization and purification	The claimed purification is in contrast to the low content of polypeptide of mol. wt. 30 000 [41]	Solubilization with cholate largely destroys the binding capacity for CAT and denatured protein

Otherwise reported solubilization and isolation procedures

The isolation of the most abundant intracellular carrier can be expected to raise such interest that a detailed analysis of methods from other laboratories seems to be warranted. We have reexamined these procedures with respect to the solubilization, the detergents used and the intactness of the carrier protein in the resulting extracts (Table II).

The earlier report of Egan and Lehninger [48] claims solubilization by Brij 56 from rat liver mitochondria of an atractylate sensitive ATP binding protein. In repeating these extracts, we have been unable to detect carboxyatractylate binding. Moreover, it was not possible to solubilize with Brij 56 or 58 from heart or liver mitochondria the [^{3}H]carboxyatractylate · protein complex to any extent (cf. Fig. 1).

Adopting our method of solubilizing the atractylate protected protein, Brandolin et al. [12] reported partial purification by affinity chromatography from liver mitochondria. The detergent used, Emulphogen, solubilizes also in our hands the intact carboxyatractylate · protein from rat liver mitochondria as well as beef heart mitochondria, although the yield is only about 30% applying their detergent : protein ratio (not shown) (cf. also Fig. 1). These authors purified their extracts by affinity chromatography rendering proteins with a molecular weight of 15 000 and 60 000. As pointed out above, their use of atractylate is also a disadvantage. There was no follow-up to this preliminary report.

The report by Bojanowski et al. [13] on the purification of this protein from an SDS-solubilized extract by "affinity chromatography" can be scarcely reconciled with the denaturing action of SDS. In our hands SDS completely destroys the carboxyatractylate binding capability. The "purified" extracts contain an ill-defined protein composition as shown by the gel electrophoresis reported by these authors. Most important, the binding data for carboxyatractylate and ADP are meaningless since they are carried out in an unspecified manner. The antigenic specificity claimed for these extracts is not indicating any carrier specificity. It is improbable that a specific affinity interaction with the column occurred. It appears that an affinity chromatography [12,13] for the isolation of the ADP, ATP carrier is not useful and may interfere with obtaining high yields of undenatured protein.

Shertzer et al. [14] report solubilization and purification of the adenine nucleotide carrier using cholate extraction and ammonium sulfate fractionation for reconstituting transport in liposomes. We had reported previously [9] that a preparation according to this procedure has a high binding capacity for carboxyatractylate, much in excess of that in mitochondria, which was due to exposure of unspecific cation-binding sites. In fact, solubilization by cholate or deoxycholate splits the native carboxyactractylate · protein complex. The gel electrophoretic analyses of these preparations show [41] that the 30 000 polypeptide protein is not enriched as compared to sonic particles so that the claim for purification is not substantiated.

In conclusion, our solubilization and purification procedure for the carboxyatractylate binding protein, as reported in detail in this paper, cannot easily be replaced by another method since simplicity, very high yield and preservation of the native state are mutually conditioning each other.

Discussion

Ligand protection

The principle of isolating the ADP, ATP carrier protein as a ligand-(inhibitor) · protein complex is without precedent in the isolation of membrane proteins. The noncovalent ligand does not only afford the demonstrated protection against denaturation, but also gives a quantitative assay for the intact protein share. Isolation of membrane protein by affinity chromatography should theoretically also take advantage of ligand protection. However, similar as in numerous other cases, affinity chromatography has not lived up to its glamorous image, in the case of the ADP, ATP carrier protein too.

The carboxyatractylate binding can be considered to maintain the protein in a defined conformation state. This is a major condition for the unusually simple isolation procedure which profits from the non-adsorbance of the solubilized carboxyatractylate · protein complex on hydroxyapatite whereas the denatured form will be adsorbed. It can be visualized that in the native state the protein exposes a large hydrophobic surface onto which Triton X-100 forms a micellar shell that prevents adsorption to hydroxyapatite, different from most other mitochondrial membrane proteins. This is consistent with the strong hydrophobicity of the carboxyatractylate · protein as indicated by the difficult extractability with detergents. In fact, one would expect from its function that the carrier protein exposes a large surface to the lipids since it must penetrate through the membrane.

Amounts in the membrane and cells

The isolation is facilitated by the large abundance in the membrane. As calculated from the carboxyatractylate content in the isolated protein and in the beef heart mitochondria (see Table I) the carboxyatractylate · protein amounts to 9% of the total mitochondrial protein and to about 12% of membrane protein. Thus the ADP, ATP carrier protein represents the major single polypeptide not only of mitochondria but also is the major membrane protein of total cells. Therefore, in the meantime according to the same procedure the carboxyatractylate · protein complex could be isolated and purified from other sources such as liver, kidney [27], Neurospora crassa (Hackenberg, unpublished and ref. 49).

The surprisingly large abundance can be rationalized considering following aspects: the protein catalyzes a transport which is the most active one in many cells. On the other hand, the turnover of the carrier site is relatively small [1] and this deficiency is made up for maintaining a high density of transport catalysts in the membrane. The transported substrate is large as compared to most other transported substrates such as cations, tricarboxylic acid cycle intermediates, glucose, etc. This may explain the relative "inefficiency" of the ADP, ATP carrier, which has to "squeeze" a much larger molecule through the membrane as most of its counterparts.

It is not surprising that such abundant protein has been isolated before, however, withouth knowledge of its identity and therefore without retaining the nature form. One example is the polypetide extracted with lysolecithin by Capaldi et al. [29] and by Harmon [32]. These proteins were given a mole-

cular weight of 29 000 or 30 000, however, no function could be assigned. More recently Boxer [30] reported that by lactoperoxidase-mediated iodination a major mitochondrial protein with a molecular weight of 29 000 was preferentially labelled. This protein has been identified with the carboxyatractylate-binding protein [31]. It is possible that a large part of "structural protein from mitochondria" [33] is also a denatured carboxyatractylate · protein.

Purity

The question may be posed whether the relatively large amounts of isolated apparently homogenous carboxyatractylate · protein are really pure or contain other polypeptides also exposing a molecular weight of 30 000 by SDS gel electrophoresis.

Some of the candidates may be β-hydroxybutyrate dehydrogenase, cytochrome *b* [34] and c_1 [35,36], the hydrophobic subunit of oligomycin-sensitive ATPase [37]. β-Hydroxybutyrate dehydrogenase with a molecular weight of about 32 000 accounts for only 0.4% of the protein of beef heart mitochondria [38,39]. Cytochrome c_1 can be excluded because no heme was detected in the preparation. Cytochrome *b* can lose the heme in Triton X-100 solution so that the apoprotein might be a concomitant. However, when cytochrome *b* was protected by antimycin A, the holocytochrome *b* does not appear in the pass-through on hydroxyapatite [20].

The oligomycin-sensitive ATPase preparations from beef heart contain a hydrophobic subunit of 29 000 in varying amounts (cf. ref. 40). The function of this subunit is not clear. It has been discussed whether it originates from the carboxyatractylate-binding protein and is a contaminant of the ATPase preparation [41]. If this subunit is different from the carboxyatractylate-binding protein, a small cross contamination in the isolated carboxyatractylate preparation is possible. One possibility to check for these contaminations would be to use antibodies against these contaminants. It may be added that in our current work on the amino acid sequence the polypeptide appears to be largely homogenous (Babel, W., unpublished).

Molecular weight

Interesting is the comparison of the molecular weights as determined by SDS gel electrophoresis and calculated from the carboxyatractylate binding, which suggests, that there are two 30 000 subunits for one carboxyatractylate-binding site. In other words, the carboxyatractylate · protein complex may be a dimer of 60 000 mol. wt. It may be noted that also a dimer structure for another transport protein, the isolated ($Na^+ + K^+$)-ATPase, has been proposed [42,43].

The identity of the isolated carboxyatractylate-binding protein with the ADP, ATP carrier must be questioned if one assumes that carboxyatractylate binds to another regulatory subunit than ADP. This suggestion has been made by Winkler et al. [44], and Vignais [45], whereas from our viewpoint the binding sites for carboxyatractylate and ADP are identical [1,46]. The large proportion of 12% of the carboxyatractylate-binding protein renders it unlikely that the membrane contains another catalytic transport subunit which should have a similarly high content. The isolated carboxyatractylate-binding protein still has a highly specific recognition site for ADP and ATP, similar as is known

for the ADP, ATP carrier in situ. We consider this as a strong argument for the identity of the carboxyatractylate-binding protein with the ADP, ATP carrier, although the protein cannot tightly bind ADP or ATP after the removal of carboxyatractylate.

Detergents and intactness

The isolation of the intact carboxyatractylate · protein complex also gives a hitherto rare opportunity to examine the ability of various detergents to extract membrane proteins in the native state. The nonionic polyglycol type detergents proved to serve best this purpose whereas cholate, often regarded as a mild detergent, is already denaturing the carboxyatractylate · protein. As reported elsewhere [19], aminoxide type reagents also solubilize the intact carboxyatractylate · protein complex, although there is evidence that these detergents are somewhat harsher. The difference in the solubilizing power of the different polyglycol detergents used may be linked to the critical micelle concentration, as the best solubilizing reagent Triton X-100 has a critical micelle concentration = 0.25 mM, whereas Brij 58 0.06 mM [47].

The protection of the protein by carboxyatractylate binding against proteolytic degradation is a very impressive example of how in a membrane the release of the intact conformation can lead to rapid proteolytic degradation. It is clearly shown that the unfolding and loss of antigenicity against the carboxyatractylate protein antibody, precedes the proteolytic degradation. Total degradation may follow actually the splitting of the dimer to the monomer. As reported elsewhere [23], when the protein is isolated as the bongkrekate protein complex, it becomes degraded already in the presence of bongkrekate to some extent. This is in accordance with the fact that bongkrekate binds less tightly than carboxyatractylate to the protein.

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References

1 Klingenberg, M. (1976) in The Enzymes of Biological Membranes: Membrane Transport (Martonosi, A.N., ed.), Vol. 3, pp. 383—438, Plenum Publishing Corp., New York
2 Klingenberg, M., Pfaff, E. and Kröger, A. (1964) in Techniques for Studying Kinetics in Mitochondrial Suspensions: Rapid mixing and Sampling Techniques in Biochemistry, pp. 33—337, Academic Press, New York
3 Weidemann, M.J., Erdelt, H. and Klingenberg, M. (1970) Eur. J. Biochem. 16, 313—335
4 Klingenberg, M., Falkner, G., Erdelt, H.and Grebe, K. (1971) FEBS Lett. 16, 296—300
5 Vignais, P.V., Vignais, P.H. and Defaye, G. (1973) Biochemistry 12, 1508—1518
6 Klingenberg, M., Grebe, K. and Scherer, B. (1975) Eur. J. Biochem. 52, 351—363
7 Babel, W., Aquila, H., Beyer, K. and Klingenberg, M. (1976) FEBS Lett. 61, 124—127
8 Lauquin, G.J.M. and Vignais, P.V. (1976) Biochemistry 15, 2316—2322

9 Klingenberg, M., Riccio, P., Aquila, H., Schmiedt, B., Grebe, K. and Topitsch, P. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., et al., eds.), pp. 229—243, North Holland Publishing Co., Amsterdam
10 Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 129—133
11 Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133—138
12 Brandolin, G., Meyer, C., Defaye, G., Vignais, P.M. and Vignais, P.V. (1974) FEBS Lett. 46, 149—153
13 Bojanovski, D., Schlimme, E., Wang, C.S. and Alaupovic, P. (1976) Eur. J. Biochem. 71, 539—548
14 Shertzer, H.G. and Racker, E. (1976) J. Biol. Chem. 251, 2446—2452
15 Riccio, P., Scherer, B. and Klingenberg, M. (1973) FEBS Lett. 31, 11—14
16 Smith, A.L. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), Vol. X, pp. 81—86, Academic Press, New York
17 Kröger, A. and Klingenberg, M. (1966) Biochem. Z. 344, 317—336
18 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656—3661
19 Krämer, R., Aquila, H. and Klingenberg, M. (1977) Biochemistry 16, 4949—4953
20 Riccio, P., Schägger, H., Engel, W.D. and von Jagow, G. (1977) Biochim. Biophys. Acta 459, 250—262
21 von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M. and Kolb, H.J. (1977) Biochim. Biophys. Acta 462, 549—558
22 Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B.B. and Grebe, K. (1976) in The Structural Basis of Membrane Function (Hatefi, Y. and Djavadi-Ohaniance, L., eds.), pp. 293—311, Academic Press, New York
23 Aquila, H., Eiermann, W., Babel, W. and Klingenberg, M. (1978) Eur. J. Biochem., in press
24 Ackers, G.K. (1970) in Advances in Protein Chemistry 24 (Anfinsen, C.B., et al., eds.), pp. 343—446, Academic Press, New York
25 Capaldi, R.A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 930—932
26 Buchanan, B.B., Eiermann, W., Riccio, P., Aquila, H. and Klingenberg, M. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2280—2284
27 Eiermann, W., Aquila, H. and Klingenberg, M. (1977) FEBS Lett. 74, 209—214
28 Hackenberg, H. and Klingenberg, M. (1977) in press
29 Capaldi, R.A., Komai, H. and Hunter, D.R. (1973) Biochem. Biophys. Res. Commun. 55, 655—659
30 Boxer, D.H. (1975) FEBS Lett. 59, 149—152
31 Boxer, D.H., Feckl, H. and Klingenberg, M. (1976) FEBS Lett. 73, 43—46
32 Harmon, H.J., Hall, J.D. and Crane, F.L. (1974) Biochim. Biophys. Acta 344, 119—155
33 Criddle, R.S., Bock, R.M., Green, D.E. and Tisdale, H. (1962) Biochemistry 1, 827—842
34 Weiss, H. and Ziganke, B. (1974) Eur. J. Biochem. 41, 63—71
35 Ross, E. and Schatz, G. (1976) J. Biol. Chem. 251, 1991—1996
36 Yu, C.A., Yu, L. and King, T.E. (1974) J. Biol. Chem. 249, 4905—4910
37 Capaldi, R.A. (1973) Biochem. Biophys. Res. Commun. 53, 1331—1336
38 Menzel, H.M. and Hammes, G.G. (1973) J. Biol. Chem. 248, 4885—4889
39 Bock, H.G. and Fleischer, S. (1975) J. Biol. Chem. 250, 5774—5781
40 Senior, A.E. (1973) Biochim. Biophys. Acta 301, 249—277
41 Serrani, R., Kanner, B.I. and Racker, E. (1976) J. Biol. Chem. 251, 2453—2461
42 Kyte, J. (1972) J. Biol. Chem. 247, 7642—7649
43 Perrone, J.R., Hackney, J.F., Dixon, J.F. and, Hokin, L.E. (1975) J. Biol. Chem. 250, 4178—4184
44 Winkler, H.H. and Lehninger, A.L. (1968) J. Biol. Chem. 243, 3000—3008
45 Vignais, P.V., Duee, E.D., Vignais, P.M. and Huet, J. (1966) Biochim. Biophys. Acta 118, 465—483
46 Klingenberg, M., Buchholz, M., Erdelt, H., Falkner, G., Grebe, K., Kadner, H., Scherer, B., Stengel-Rutkowski, L. and Weidemann, M.J. (1972) in Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G.F., et al., eds.), pp. 465—486, Academic Press, New York
47 Becher, P. (1966) in Nonionic Surfactants (Schick, M.J., ed.), pp. 478—515, Marcel Dekker, Inc., New York
48 Egan, R.W. and Lehninger, A.L. (1974) Biochem. Biophys. Res. Commun. 59, 195—201
49 Klingenberg, M., Aquila, H., Riccio, P., Buchanan, B.B., Eiermann, W. and Hackenberg, H. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., et al., eds.), pp. 431—438, North-Holland Publishing Co., Amsterdam